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Parkinson's disease (PD)
results in part from the loss of dopamine (DA) neurons. We hypothesize that exercise reduces the vulnerability of DA neurons to neurotoxin exposure and have outlined experiments to test this hypothesis in rats treated with one of several neurotoxins, beginning with 6-hydroxydopamine (6-OHDA). Over the past year, we established a staff, the training, and most of the methodology needed to perform these studies. Subsequently, we have observed the following: (1) Casting protects against the effects of 6-OHDA administered along DA axons or in terminal fields. (2) This protection appears to result from the blockade of DA neuron degeneration. (3) There is little or no protection against the neuropathological and behavioral effects of 6-OHDA with treadmill running using the paradigms examined. (4) Exposure to very low levels of 6-OHDA results in significant protection against higher levels of 6-OHDA exposure. We have three primary objectives for the coming year: to complete our attempts to protect against 6-OHDA toxicity with treadmill running, explore other forms of exercise, and use an effective form of exercise to examine the relation between exercise duration and protection as well as the temporal relation between exercise, time of toxin exposure and protection.

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Introduction:

Parkinson's disease (PD) results in part from the progressive loss of dopamine (DA) neurons projecting from substantia nigra (SN) to striatum. Although the cause of this neurodegenerative process is unknown, candidates include oxidative stress, mitochondrial dysfunction, and excitotoxicity. These are likely to result from exposure to environmental toxins, perhaps coupled with one or more bases for increased vulnerability. Such increased vulnerability could include genetic predisposition, emotional or physical stress, or exposure to certain recreational drugs. We have developed the hypothesis that exercise *reduces* the vulnerability of DA neurons to neurotoxin exposure under basal conditions and blocks stress-induced exacerbation of toxin-induced DA neuron loss.

In this series of studies, adult male rats are to be given one of several exercise regimens prior to toxin exposure. Behavioral, biochemical, and histological analyses will be used to determine (1) whether the neuroprotective effects of cast-induced limb use in rats treated with 6-hydroxydopamine (6-OHDA) are also seen with other forms of exercise; (2) how much exercise is required, when must it occur, and how permanent are the effects; (3) if exercise also protects against the increased vulnerability to toxins caused by other stressors; and (4) the generality of our results with 6-hydroxydopamine to other toxins.

Body:

1. Recruitment and training of staff

Our initial objective was to recruit and train a staff to carry out this study. Immediately after obtaining funds (June 2003) we hired a research assistant, Michelle Fischer, who has taken the lead in data collection. In addition, we recruited three postdoctoral fellows, Amina El Ayadi (August 2003), Rehana Leak (January 2004), and Niklas Lindgren (January 2004) who are spending a portion of their time on this grant. Each of these individuals has now been trained to do small animal stereotaxic surgery, carry out neurological tests on rodents, and to analyze the loss of dopamine (DA) neurons using both immunocytochemistry (ICC) and high performance liquid chromatography (HPLC). We also have developed the necessary expertise to quantify the loss of DA terminals and cell bodies using densitometry and stereology, respectively. This required about 4 months.

2. Effect of physical therapy on the neuroanatomical response to 6-OHDA:

Animals exposed to unilateral infusion of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB) – the path taken by DA axons from the ventral mesencephalon to their targets in the corpus striatum – show an extreme motor asymmetry in their use of the affected limb and a unilateral loss of striatal DA. Just prior to the beginning of the current 4-year period of funding, we had shown that these effects

can be blocked by placing a cast on the forelimb ipsilateral to the infusion of 6-OHDA for 7 days immediately before surgery *(1)* or after it *(2)*.

As a joint project between this grant and a grant from the National Institute of Neurological Disorders and Stroke (NINDS), we have begun to explore the question of whether exercise spares DA cells from 6-OHDA-induced toxicity or promotes the subsequent sprouting of residual neurons. Animals had one forelimb casted for 7 days prior to infusion of 6-OHDA into the ipsilateral MFB. The casts were removed prior to the surgical procedure, 6-OHDA administered, animals sacrificed on day 2 or 7 post-lesion, and brains harvested. Tissue then was analyzed by ICC for tyrosine hydroxylase immunoreactivity (TH^+) (a measure of the DA phenotype), Fluoro-jade B staining (a marker of degeneration), and Nissl immunoreactivity (a measure of cell number). Seven days after being given 6-OHDA, animals with no prior casting showed ~60% loss in TH^+. This was preceded by a 340% increase in Fluoro-jade staining. In marked contrast, animals with casting for 7 days prior to infusion of 6-OHDA showed a <20% loss of striatal TH^+ by day 7 and *virtually no increase* in Fluoro-jade-immunoreactivity compared to sham-lesioned animals either 2 or 7 days post-op.

In the SN, 6-OHDA-treated animals with or without casting showed a comparable decrease (20%) in TH^+ at 2 days post-lesion that was concomitant with a ~20% decrease in Nissl immunoreactivity, indicating that some actual cell loss had occurred. By day 7, a further 55% decrease in TH-immunoreactive cells in the SN had been lost in 6-OHDA-treated animals. In contrast, *no further decrease in TH was observed in animals casted prior to 6-OHDA infusion.* These results – coupled with our previous observation that casting prevented the 6-OHDA-induced loss of striatal DA over a period of more than a month – strongly suggest to us that forced exercise prior to infusion of 6-OHDA greatly reduces the vulnerability of DA neurons to the toxin. Ongoing experiments will test the hypothesis that this represents a blockade of degeneration rather than a delay. This study has involved approximately 90 animals. The study is almost complete and will be reported at the 2004 meeting of the Society for Neuroscience and prepared for publication. It also will be extended to examine non-motor projections of DA neurons, such as the projection from VTA to nucleus accumbens.

3. *Effect of treadmill running on the 6-OHDA toxicity in the MFB.*

In Task 1 of the Statement of Work, we proposed to determine if the protective effects of forced limb use would generalize to other forms of exercise, such as treadmill running and swimming. The initial form of exercise in which we focused was treadmill running. A group of animals was initially subjected to 7 days of treadmill running, the same number of days we had shown to be protective when casting was used to force contralateral limb use. Two groups of animals were trained to run on the treadmill for 3 days. If animals refused to run, they received a mild shock, standard practice for treadmill studies. After this training period, rats received a 4-day rest period. Trained animals were then run for 45 min/day at 17 m/min for 7 days or were placed on the treadmill for the same time period with the treadmill in the off mode. Control animals were placed on the treadmill in the off position during the training and the running mode. Immediately following the last running session, all animals received 3 μg 6-OHDA into the MFB.

Animals were sacrificed 7 days after infusion of 6-OHDA, and the striatum was dissected and assayed for DA using HPLC. Animals given 6-OHDA without being casted had a 70% DA depletion compared to the contralateral striatum. To our surprise, this depletion was *increased* by 30% in animals that were run on a treadmill for 7 days prior to infusion of 6-OHDA. Since we had previously observed that stress can exacerbate the neurotoxic effects of our lesion, this suggested that the treadmill paradigm we were using was too stressful.

During the time we were attempting to develop our own treadmill protocol, Dr. Jennifer Tillerson published a paper *(3)* indicating that short bouts of treadmill running protected against 6-OHDA toxicity in the rat. Therefore, we examined the impact of a modified version of our paradigm that was more analogous to that used by Tillerson and her colleagues. Animals were again trained to run on the treadmill for 3 days. However, we abolished the rest period and altered the running schedule. Beginning on day 4, animals were run twice a day for 15 min at 17 m/min for 7 days, with the running sessions separated by 3 hrs. Control animals were placed on the treadmill in the off position during the training and the running period. Immediately following the last running session, all animals received an infusion of 0.5 μg 6-OHDA into the MFB. Despite these modifications, there was no effect of treadmill running on forelimb placement or amphetamine induced rotations and only a small (25%) non-significant decrease in lesion size in animals that ran prior to infusion of 6-OHDA. This study has involved approximately 40 animals.

4. *Effect of different forms of exercise on a progressive 6-OHDA lesion model.*

a. Forced limb-use: Parkinson's disease is a progressive neurodegenerative disease whereas the degeneration observed in the MFB 6-OHDA lesion model of parkinsonism is very rapid (cell death in ≤ 7 days). Thus, we wanted to examine the efficacy of forced-limb use in a more progressive rodent lesion model where 6-OHDA is infused into the striatum rather than the MFB. Under these conditions, the death of DA neurons in the SN is protracted, occurring over months post-infusion of 6-OHDA. Animals were infused with 16 μg 6-OHDA directly into the striatum. Two hours after surgery, while the animals were still anesthetized, a cast was placed on the ipsilateral forelimb. The cast was removed 7 days after infusion of 6-OHDA, animals perfused, and the SN and striatum sectioned and analyzed for TH^+ by ICC. As observed with the MFB lesion model, animals that were forced to use the contralateral forelimb for 7 days after infusion of 6-OHDA into the striatum were completely protected against 6-OHDA induced toxicity. This study has involved 9 animals.

b. Treadmill running: As mentioned earlier, infusion of 6-OHDA into the striatum produces a progressive degeneration of the nigrostriatal pathway that occurs over months. Additionally, 2-4 weeks of treadmill running has been shown to protect against ischemic damage in the brain *(4, 5)* and damage against domoic acid and 3-acetylpyridine *(6)*. Thus, in the next experiment animals were trained for 3 days on the treadmill and then forced to run for 5 day/wk at 15 m/min for 30 min for 2 weeks. At the end of the 2 week running period, animals received an infusion of 16 μg 6-OHDA into the striatum. After a 3 day recovery period, animals were run on the treadmill for 4 days,

perfused, striatum sectioned and assayed by ICC for TH^+. Unfortunately, because of the large variability in lesion size, no definitive conclusion could be drawn and the study will have to be repeated. It involved 12 animals.

5. Preconditioning with toxin exposure

It has been shown in animal models of stroke and cardiac disease that exposure to small stressors for short durations can confer resistance to subsequent stressors in a variety of organs. Although not proposed in our original application, we felt that it would be instructive to determine if exposure to low levels of toxin could also cause "preconditioning" in a model of Parkinson's disease. Thus, a very low dose of 6-OHDA (0.5 – 1.0 μg) was infused into the striatum and four days later this was followed by a much higher dose (16 μg.) After a 7-day interval, rat brains were perfused and TH^+ was assessed. The area of TH^+ loss was reduced by 30% in "preconditioned" animals relative to animals receiving only the larger dose of 6-OHDA (4.9 ± 0.4 mm^2 versus 3.5 ± 0.4 mm^2). This study has thus far involved 12 animals.

Key Research Accomplishments:

- Hired one research assistant and three postdoctoral fellows who will contribute to this research program.
- Provided training in small animal handling and biosafety techniques (required certifications), stereotaxic surgery, behavioral analysis, ICC, and HPLC.
- Developed methods for analyzing the neuroanatomical effects of the application of neurotoxins on terminals (densitometry) and cell bodies (stereology).
- Established a second model for PD, one in which the 6-OHDA is applied to the terminals of DA neurons within the striatum rather than their axons along the MFB. (This results in a progressive loss of neurons rather than the much more rapid effects of traditional MFB lesions.)
- Developed several protocols with which to examine the impact of treadmill running on vulnerability to neurotoxins.
- Developed a protocol for looking at the impact of very low levels of toxin exposure on the effects of subsequent higher levels of exposure.

Reportable Outcomes:

- Casting protects against the neuropathological and behavioral of 6-OHDA administered in both the MFB and striatum of rats, models that normally produce loss of DA and a variety of motor deficits.
- This protection appears to result from the blockade of DA neuron degeneration rather than sprouting, regeneration, or neurogenesis.

- There is little or no protection against the neuropathological and behavioral effects of 6-OHDA with treadmill running using the paradigms examined.

- Exposure to very low levels of 6-OHDA (0.5-1.0 μg) results in significant protection against higher levels of 6-OHDA exposure (16 μg).

Conclusions and Plans for Year 02:

We have had difficulty in developing a treadmill-running paradigm that protects against 6-OHDA toxicity using both the MFB and striatal infusion models. Although Tillerson et al., 2003 *(3)* reported protection against 6-OHDA toxicity with small bouts of running starting 2-4 hours after surgical infusion of 6-OHDA, in our initial attempt to replicate the Tillerson results it appeared that the animals had not recovered sufficiently from surgery to allow them to run on the treadmill. Indeed, a 3-day recovery period generally was needed for the animals to reliably run on the treadmill. Yet, with the inclusion of this recovery period, there was only a *trend* towards protecting against 6-OHDA toxicity. We are in the process of repeating this experiment.

Separation of animals based on running performance: In the above studies, we did notice that animals varied in their acquisition of running on the treadmill. Specifically, there appeared to be two populations of animals — "good runners" and "bad runners." Rats are known to differ in their response to a novel environment (e.g. high responders versus low responders) and this response correlates with the extent of activation of the HPA axis *(7-10)*. This response to novelty can be used to predict an animal's propensity to self-administer psychostimulants *(11)* and its response to stressors *(9, 10, 12)*. Further, high responding and low responding rats also display differences in the extent of neurogenesis in the dentate gyrus *(13)*. Thus, it may be that the "good runners" and "bad runners" differ in their response to the effects of treadmill running, and that this difference underlies the variability that was observed in lesion size. In our next experiment with a treadmill, we will categorize the rats based on their acquisition of treadmill running and then see if this helps to predict the impact of this form of exercise.

Use of running wheels: On the other hand, although treadmill running has been shown to protect against ischemic insults and has been used successfully in one study of PD models, we are beginning to doubt its value to us. If nothing else, there are simply too many variables to be examined – speed, duration, sessions per day, number of days, and so forth. Thus, we are seeking alternative approaches. In this regard, voluntary running now seems to us to be a more promising approach. First, use of a running wheel has been shown to increase neurotrophic factors in the brain *(14-16)*, to induce neurogenesis *(17, 18)* and protect against various brain insults *(19)*. Second, unlike treadmill running, which cannot properly be called a *voluntary* form of exercise, rodents choose to run in a wheel if it is attached to their cage.

Therefore, in the first half year ahead we will to explore the use of a voluntary wheel running as an alternative to casting, which works but is not a practical approach to promoting exercise, and to treadmill, which may well act at least as much as a stressor than as exercise (Task 1). In order to do so, we are requesting permission to rebudget

funds to permit the purchase of the necessary running wheel units. Other approaches may also be explored. The remainder of the year will use the most effective form of exercise to focus on Task 2, examining the relation between exercise duration and protection as well as the temporal relation between exercise, time of toxin exposure and protection.

Preconditioning: We believe that we now have evidence that exposure of animals to low-doses of toxins "inoculates" or "preconditions" them against the effects of high doses of toxins. If our program officer agrees that this is a potentially important phenomenon, we will repeat our initial experiment and extend it to determine whether exposure of animals to a low dose of one type of toxin can product against others. We also will ask how long the protection lasts.

Non-motor effects of exercise: There is an increasing awareness that Parkinson's disease involves much more than motor symptoms. Thus, although this was not described in our original Statement of Work, we would like to expand our battery of behavioral tests to include tests of cognitive function and mood, and also to examine DA projections other than the nigrostriatal projection (e.g., the projection from VTA to nucleus accumbens.

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APPENDICES:

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